Multivalent Polymer-Peptide System Containing Eye Drops for Enhanced Hyaluronic Acid Retention on Ocular Surfaces

by
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ABSTRACT

Dry eye disease is an ocular condition that affects millions of people in the United States alone, especially the elderly population. Current treatment methods for mild cases of the disease are saline-, polymer-, or hyaluronic acid (HA)-based eye drops. Recent findings show that HA-based eye drops are more effective. In addition, HA is known to act as a natural lubricant. However, one of the major problems with current eye drop technology is that they need to be reapplied frequently due to blinking of the eye. Thus, an HA binding eye drop technology that prolongs HA retention on the ocular surface would benefit people who experience dry eye symptoms and therefore need an effective treatment method for smooth blinking with sufficient hydration. Recently, a biofunctional polymer-peptide system was reported that enhanced HA retention on ocular surfaces. This system has one peptide that binds to HA and another peptide that binds to the ocular surface via transmembrane mucins. Here, we investigate a multivalent polymer-peptide system that further enhances HA retention due to multivalent peptides. We harnessed a system that comprises of an inclusion complex (IC) of alpha-cyclodextrin/poly(ethylene glycol) or PEG. α-CD is a 6-membered ring shaped oligosaccharides with a molecular cavity and has several hydroxyl groups available for further chemical modifications. Since, α-CD can thread on PEG chains, the IC provides multi-valency through hydroxyl groups of α-CD for multi-peptide attachment. This multivalent approach allows HA to be immobilized on the ocular surface, thus allowing for longer retention time due to an overall higher binding affinity. We synthesized PEG/CD IC and end-capped PEG chains. We further modified hydroxyl groups of α-CD into carboxylic acids to enhance its aqueous solubility. The carboxylic acid groups were further modified to acrylate

functionality. The thiol-containing mucins- and HA-binding peptides were linked to the PEG/CD complex via thiol-acrylate Michael-addition reaction. We applied the resultant multivalent polymer-peptide system with HA on *ex vivo* rabbit eyes and studied HA retention overtime. We also compared the HA retention with a 2-arm and an 8-arm polymer-peptide system. Through *ex vivo* rabbit studies, this novel eye-drop technology of a multivalent polymer-peptide system retains ~1.2 times more HA than the 8-arm polymer-peptide system, ~1.3 times more HA than the PEG/CD complex without HA binding peptide, and ~1.5 times more HA than a commercially available HA-based eye drop. Through our preliminary *in vivo* mouse studies, this new eye drop technology showed an ocular surface residence time of ≤ 30 minutes as compared to ≤ 15 minutes for the 2-arm and 8-arm polymer-peptide systems and for a commercially-available HA-based eye drop. Thus, we anticipate that a multivalent HA-binding eye drop technology has great potential to be an effective treatment method for dry eye disease.

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PREFACE/ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1 The ocular surface and dry eye disease

The ocular surface is the front-most outer surface of the human eye, separating the eye from the environment. It is made up of the cornea, conjunctiva, and the tear film, and is shown in **Figure 1**. Its main function is to protect the eye from injury and infection. The cornea is a clear tissue that acts as the eye's outermost lens by controlling and focusing light into the eyes. The conjunctiva is a thin, transparent, mucous layer that covers the front of the sclera (or the white part of the eyes) and lines the insides of the eyelids.[1] The tear film covers the cornea and is the eye's first defense to the environment. **Figure 1** also shows the three layers of the tear film: the mucin layer, the aqueous layer, and the lipid layer (from the innermost to the outermost layer). The mucin layer coats the cornea and acts as an anchor, holding the tear film to the eye. The aqueous layer is the watery layer that makes up the majority of our tears. The lipid layer helps keep tears from spilling out of the eyes or evaporating from the surface of the eyes.[2,3,4]

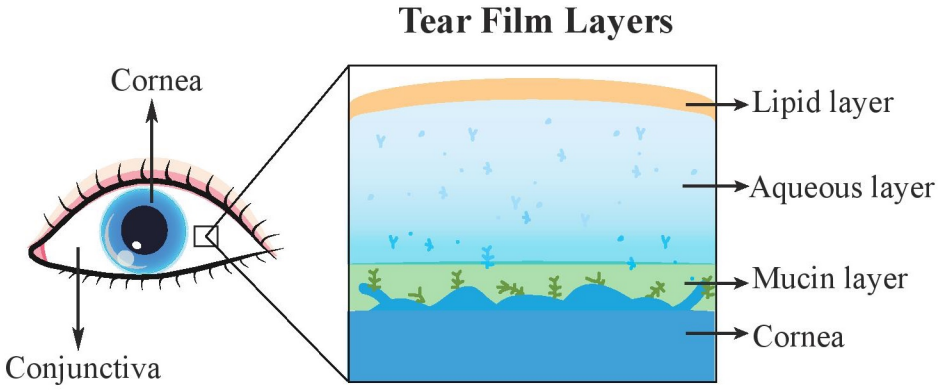


FIGURE 1. Diagram of the ocular surface components and the tear film layers.

Dry eye is an ocular condition prevalent in the elderly population, but affects thousands of people on a day-to-day basis. Although dry eye is a multifactorial disease, it occurs mostly due to any alterations in the tear film composition and stability with potential damage to the ocular surface. There are two types of dry eye: aqueous-deficient and evaporative. Aqueous-deficient dry eye is caused by the tear film's inability to produce sufficient tear fluid, whereas evaporative dry eye is caused by increased tear evaporation rate because of damage to the lipid layer in the tear film.[5] Symptoms include dryness or lack of hydration, discomfort, and visual disturbances and pain in the eye. About 40% of Americans have reporting experiencing dry eye symptoms.[6] There have been many different approaches to treating dry eye symptoms, most involving the application of a saline or polymer-based eye drop to reduce discomfort and increase water retention on the ocular surface. About 1 out of 5 adults report using over-the-counter eye drops to treat symptoms at least five times per week. However, 63% of people who use over-the-counter eye drops report that these drops are not sufficiently effective to reduce the symptoms.[6] It is mostly due to the temporary nature of the treatment, and these artificial tears must be reapplied frequently because of their short residence time on the eye.

1.2 Current treatment method

Currently, the main treatment method for mild cases of dry eye symptoms is application of eye drops. Throughout the history of eye drops, there have been mainly three generations of eye-drop compositions. The first generation of modern artificial tears can be traced back to the product, Collyrium, which means "eye wash" in Latin. This

product and other early attempts at dry eye relief gave rise to saline-based eye drops; however, these artificial tears spread poorly across the ocular surface and had short retention time leading to transient relief. Around the 1980s, artificial tears advanced to integrating natural and synthetic polymers (*e.g.*, methylcellulose derivatives, poly(ethylene glycol), and polyvinyl alcohol) in eye drops. The second generation, polymer-based eye drops offered better retention time due to the structure of polymers. These polymers have high viscosity, which allowed them to stay on the eye longer than the preceding saline-based eye drops. This demonstrates a clear structure-property relationship for the use of polymers in eye drops. However, one of their major drawbacks is its occasional, transient visual blurring due to their gel-forming nature.[7] The third generation of modern artificial tears incorporates HA (**Figure 2**, chemical structure of HA), which is a naturally occurring polysaccharide, or more specifically an anionic glycosaminoglycan that is a major component of the extracellular matrix. It is a long polymeric chain with many carboxylic acid and hydroxyl functional groups. Its long chain highlights its elastic properties as a viscoelastic molecule, allowing it to bind together for long-lasting coverage. The carboxylic acid and hydroxyl functional groups allow it to form many hydrogen bonds, causing it to be extremely hydrophilic and allowing it to prolong water retention.[8] HA has been shown to ameliorate dry eye symptoms due to its viscoelastic nature, leading to its ability to prolong water retention, tear film stability, and surface lubrication,[9] as well as reduce tear evaporation and permit uninterrupted blinking.[10] In addition, HA has a number of desirable therapeutic properties, for example, it encourages corneal wound healing by promoting epithelial cell migration[11] and reduces inflammation.[12] Recent studies further indicate that anionic

polysaccharide moieties, including HA, can enhance the spreading of the tear film lipid layer, which is an added clinical benefit of HA for ocular application.[13] It has also been demonstrated that HA can be beneficial for patients with both aqueous deficient and evaporative dry eye because it increases tear volume and tear film stability.[14] In the market today, there are commercially available eye drops containing 0.1-0.2% HA, such as *Opticalm*, *Scope*, *Oxyal*, and *Hylo*. Even though this third generation HA-based eye drop effectively wets and lubricates the ocular surface, it still has a low residence time overall of about <10 min, which requires frequent application.[15]

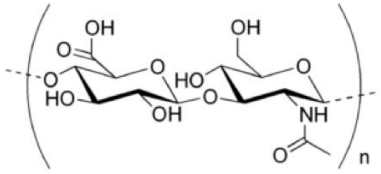


FIGURE 2. Chemical structure of HA.

Compared to the other eye drops, 0.2% HA has a longer mean half-life in artificial tears solutions of 321 seconds on the ocular surface, as compared to 0.3% hydroxyl propyl methylcellulose's (HPMC) half-life of 44 seconds and 39 seconds of 1.4% poly(vinyl alcohol) (PVA).[16] HA concentrations of 0.1% and 0.3% have been shown to delay tear break up time (TBUT), an important parameter that reflects tear film stability. Compared to the polymer-based eye drops containing HPMC or PVA, 0.1% HA has been shown to be superior in stabilizing the pre-corneal tear film.[17] HA is also superior to HPMC and PVA for water retention and protection of human corneal epithelial cells from dehydration.[18] The major limitation for current eye drop technology is its low retention time on the ocular surface. Thus, an eye drop technology that effectively binds HA to the

ocular surface is needed to enhance its retention time, which potentially can eliminate the necessity of frequent eye-drop instillation.

1.3 Multivalent polymer-peptide system

Our overall objective is to enhance the binding of HA to the ocular surface. This would help retain a thin film of moisture on the ocular surface to mimic the smooth, hydrated, and lubricated natural surface. As mentioned previously, the mucin layer of the tear film acts as an anchor, holding the tear film to the eye. Thus, theoretically, binding HA to a component of the mucin layer would allow for the optimal and longest retention. Mucins are high-molecular weight glycoproteins with a protein backbone and high carbohydrate content. There are two types of mucins in the tear film: membrane-bound and secreted. The membrane-bound mucins are embedded in the lipid bilayer of the mucin layer, while the secreted mucins are released into the extracellular environment of the aqueous layer.[19] Membrane-bound mucins such as mucin-1, mucin-16, and mucin-20 contain sialic acid in the *O*-glycosylated extracellular domains.[20] Sialic acid is a family of acidic monosaccharides, and it is usually found at the outermost of the glycan chains on the cell surface proteins and lipids.[21] Therefore, given the location of sialic acid on the membrane-bound mucins in the mucin layer of the tear film, there will be easier access to sialic acid, allowing sialic acid to be harnessed as the potential anchoring site for immobilizing HA on the ocular surface. Current research shows that a certain sialic acid binding peptide (or SABpep) binds to sialic acid well, with a strong binding affinity[22]; therefore, SABpep (GGSPYGRC) was chosen as the mucin anchoring peptide and HABpep (STMMSRSHKTRSHHVGC) to bind HA.[22]

Polyethylene glycol (PEG) was chosen as the space-polymer because of its properties that make it especially useful in various biological, chemical and pharmaceutical settings. PEG is the current ideal polymer to conjugate with proteins due to its non-toxic and non-immunogenic, hydrophilic/aqueous/soluble, and highly flexible properties. It can be added to media or conjugated to molecules without interfering with cellular functions or target immunogenicities. When attached to proteins, it decreases protein aggregation and increases solubility. Since it is a linear chain, its highly flexible nature provides for bioconjugation without steric hindrance.[23] **Figure 3** shows the structure of PEG, which has hydroxyl groups at the two ends of the long chain molecule, allowing it to form hydrogen bonds, and thus making it hydrophilic. This particular characteristic of its structure allows it to increase the solubility of anything that it is conjugated to. This demonstrates the structure-property relationship of PEG.

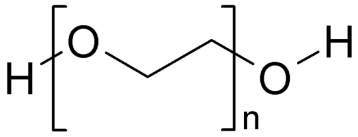


FIGURE 3. Chemical structure of PEG.

PEG can be complexed with alpha-cyclodextrin (α-CD), using supramolecular chemistry, to create an inclusion complex (IC) of PEG/CD. α-CD is a sex-member oligosaccharide with the structure of a doughnut ring with an inner cavity and an outer ring.[24] It contains many hydroxyl groups that can be easily modified to create a diverse amount of functions through different chemical reactions. Succinyl-α-CD (succ-CD) has a carboxylic acid functional group instead of one of the hydroxyl groups of α-CD, which provides a site for conjugation to attach a binding peptide that attaches HA (HA-binding

peptide or HABpep) or modified to attach SABpep that binds to sialic acid in the mucin layer. The succ-CD rings physically thread onto the PEG chains, and this allows it to resemble a molecular necklace. The succ-CD rings are incredibly mobile, and thus, can slide along and rotate around the PEG chain.[24] **Figure 4** shows the structure of succinyl-α-CD. The amino acid sequence of SABpep is GGSPYGRC. The amino acid sequence of HABpep is STMMSRSHKTRSHHVGC. Both peptides contain thiols (SH) that undergo thiol-Michael addiction reactions with enes (such as acrylate). Therefore, the PEG/CD IC with –COOH functional groups can be modified to acrylate functional groups, which can then be bound to the thiol groups in the peptides. This reaction can be seen in **Schemes 1 and 2**.

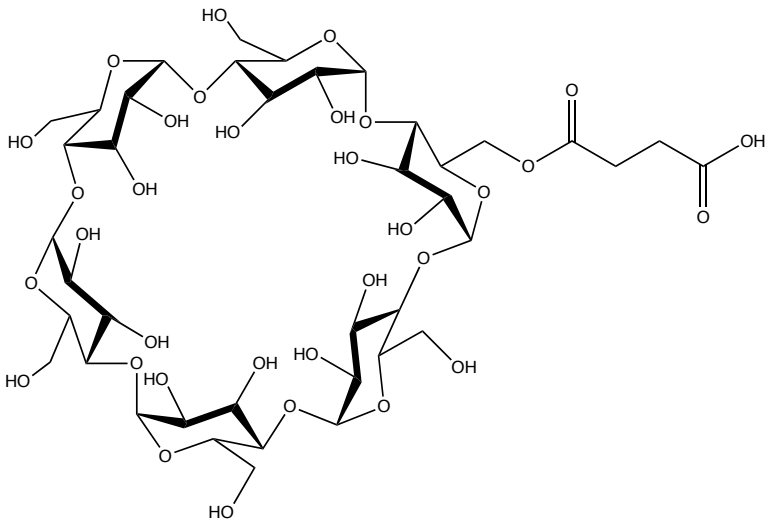
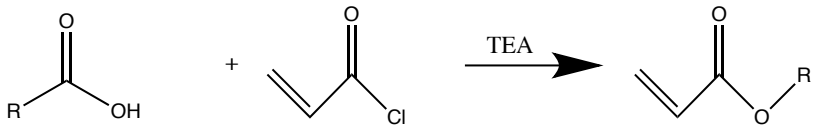
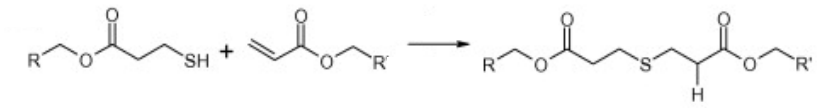


FIGURE 4. Chemical structure of succinyl-α-cyclodextrin.



SCHEME 1. Carboxylic acid (from PEG/Succ-CD) + Acryloyl chloride and TEA to yield PEG/Succ-CD/Acrylate.



SCHEME 2. Thiol (from peptides) + acrylate group (from PEG/Succ-CD/Acrylate) to yield PEG/Succ-CD/peptide.

2. MATERIALS AND METHODS

2.1 Materials

Succinyl-α-cyclodextrin (Succ-CD) (M_w 1300 Da) was purchased from Cyclodextrin-Shop (Tilburg, Netherlands). Azide-PEG-Azide (M_w 20 KDa) was purchased from Creative PEGWorks (Chapel Hill, NC). And DBCO-PEG4-Hydroxyl (M_w 508.61 Da) was purchased from Click Chemistry Tools (Scottsdale, AZ). Triethylamine (TEA) (M_w 101.2 Da) was purchased from Chem-Impex (Wood Dale, IL). Acryloyl chloride (M_w 90.51 Da) was purchased from Sigma-Aldrich (St. Louis, MO). Immobilized TCEP Disulfide Reducing Resin (or TCEP gel) (M_w 286.65 Da, concentration of 8 μmol/mL) was purchased from ThermoFisher Scientific (Rockford, IL). The sialic acid binding peptide (SABpep or GGSPYGRC) (M_w 795.86) and the hyaluronic acid binding peptide (HABpep or STMMSRSHKTRSHHVGC) (M_w 1942.21 Da) were custom synthesized and purchased from Synpeptide (Shanghai, China).

2.2 Synthesis of End-capped PEG/Succ-CD

Succ-CD (1 g, 0.769 mmol) was dissolved in 3 mL of distilled water. Azide-PEG-Azide in a 0.05 molar ratio relative to Succ-CD (50 mg, 2.5 μmol) was dissolved in 0.4 mL of distilled water. Then, the Succ-CD solution was added to the PEG solution, vortexed, and stirred overnight at room temperature. For the PEG end-capping reaction, DBCO-PEG4-Hydroxyl in a 4 molar ratio (2.54 mg, 5 mmol) was dissolved in 0.2 mL of anhydrous dimethyl sulfoxide (DMSO). This solution was then added to the PEG/Succ-CD inclusion complex, vortexed, and stirred overnight at room temperature. Then, it was dialyzed against distilled water overnight (M_w cutoff 3500 Da). Water was then removed

by lyophilization to produce end-capped PEG/Succ-CD, which was characterized by ^{1}H-NMR in DMSO (**Figure 5A**) using the Bruker Advance III 500 MHz NMR spectrometer. The Bruker TopSpin software was used to process the NMR spectrum.

2.3 Synthesis of PEG/Succ-CD/Acrylate

PEG/Succ-CD (125 mg, 0.096 mmol of Succ-CD) was dissolved in 3 mL of anhydrous dimethylformamide (DMF) and kept stirring on a stir plate. TEA was added dropwise while stirring in a 15 molar ratio to Succ-CD (201.19 μL, 1.44 μmol). Then, acryloyl chloride was added dropwise while stirring in a 15 molar ratio to PEG/Succ-CD (116.56 uL, 1.44 μmol). This mixture was left stirring overnight at room temperature, and then precipitated in chloroform and rotovaporized to remove TEA salt and DMF. Then, it was dialyzed against distilled water overnight (M_w cutoff 500-1000 Da) and lyophilized to produce PEG/Succ-CD/Acrylate, which was characterized by 1H NMR in D_2O (**Figure 5B**).

2.4 Synthesis of PEG/Succ-CD/SABpep and PEG/Succ-CD/SABpep/HABpep and 8-arm PEG/SABpep/HABpep

SABpep and HABpep were characterized by 1H NMR in D_2O (**Figure 5C and 5D**, respectively). To synthesize PEG/Succ-CD/SABpep, PEG/Succ-CD/Acrylate (15 mg, 0.0115 mmol of Succ-CD) was dissolved in 1 mL of PBS. TCEP gel (1 mL, 8 mmol) was washed with PBS 2 times, and SABpep in a 0.5 molar ratio (4.59 mg, 5.77 μmol) was dissolved in 1 mL of PBS and added to the TCEP gel and was stirred for an hour. After centrifugation to remove the TCEP gel, the reduced peptide was added to the PEG/Succ-

CD/Acrylate. To synthesize PEG/Succ-CD/SABpep/HABpep, the same protocol was used, however with another set of TCEP gel for HABpep. The reagents were: PEG/Succ-CD/Acrylate (15 mg, 0.0115 mmol of Succ-CD) in 1 mL of PBS, two sets of TCEP gel (1 mL each, 8 mmol), SABpep in a 0.5 molar ratio (4.59 mg, 5.77 μmol) in 1 mL of PBS, and HABpep in a 0.5 molar ratio (11.21 mg, 5.77 μmol) in 1 mL of PBS. Both these reactions were stirred overnight at room temperature. Then, they were dialyzed against distilled water for 4 hours (M_w cutoff 3500 Da), and lyophilized to produce PEG/Succ-CD/SABpep and PEG/Succ-CD/SABpep/HABpep, which were characterized by 1H NMR in D_2O (**Figure 5E and 5F**, respectively). To synthesize 8-arm PEG/SABpep/HABpep, the same protocol was used, however with 8-arm PEG with maleimide groups at the end to conjugate with the thiols in SABpep and HABpep.

2.5 *Ex vivo* testing of PEG/Succ-CD/SABpep and PEG/Succ-CD/SABpep/HABpep binding over 3 periods of eye drop application

Cadaveric rabbit eyes were purchased from Pel-Freez Biologicals (Rogers, AR), and the ocular surface/conjunctivas of the eyes were excised. PEG/Succ-CD/SABpep and PEG/Succ-CD/SABpep/HABpep solutions, both at 2 mg/mL concentration, were applied on the ocular surface of the rabbit eyes for 20 min. After washing twice with PBS, HA-FL, at 30 ug/mL, was applied to the same area for 20 min. The standard curve HA-FL concentrations were 0, 10, 30, 50, 75, 100, and 200 μg/mL. The over-time retention of HA on the conjunctiva tissues was examined by using Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT) to measure fluorescence values with endpoint scan starting at time t = 0 (20 min after polymer peptide application and 20 min

after HA-FL application), and periodically every 5 min for a total of 75 min. The first measurement provided a standard curve of fluorescence values at the above concentrations. Then, the tissues were washed with PBS to remove any unbound HA-FL before fluorescent reading and then measured again 15 times, with 5 min in between each wash. After the 15th wash, the polymer-peptide and HA-FL eye drop solutions were re-administered for 20 min each and then the 15 washes were repeated for the 2nd period. This was then repeated for a 3rd period. The fluorescent values of PEG/Succ-CD/SABpep (control) were then compared to the fluorescent values of PEG/Succ-CD/SABpep/HABpep (sample). All groups were performed in triplicate. This *ex vivo* study is to demonstrate the periodic effectiveness of the eye drop technology.

2.6 *In vivo* testing of PEG/Succ-CD/SABpep/HABpep binding

In vivo tests were performed on mice (C57BL/6, Charles river), and they were anesthetized with isoflurane (Baxter, IL). Six mice were used for 3 different time points, 2 mice per time point, and each eye was used for either a control eye drop or sample eye drop. Mice 1 and 2 were for 0 min time point, mice 3 and 4 were for 15 min time point, and mice 5 and 6 were for 30 min time point. The mice were split into 4 sets to compare the eye drop solutions at the 3 different time points t = 0 min, 15 min, and 30 min, as shown in **Figure 7**. The first two sets (**Figures 7A and 7B**) compare the negative control eye drop solution of mice 1, 3, and 5. The third set (**Figure 7C**) compares the positive control eye drop solution of mice 2, 4, and 6. The fourth set (**Figure 7D**) compares the sample eye drop solution of mice 2, 4, and 6. The left eyes of mice 1, 3, and 5 (1L, 3L, and 5L) were treated with an eye drop containing the negative control of PBS (total

volume 5 μL), and the right eyes of mice 1, 3, and 5 (1R, 3R, and 5R) were treated with an eye drop containing the negative control of the multivalent polymer-peptide, SABpep-PEG/CD-HABpep (without HA-FL) (total volume 5 μL, with a concentration of 2 mg/mL). The left eyes of mice 2, 4, and 6 (2L, 4L, and 6L) were treated with an eye drop containing a combined 1:1 vol ratio of PBS and 1 mg/mL of HA-FL (total volume 5 μL), and the right eyes of mice 2, 4, and 6 (2R, 4R, and 6R) were treated with an eye drop containing a combined 1:1 vol ratio of 2 mg/mL SABpep-PEG/CD-HABpep and 1 mg/mL of HA-FL (total volume 5 μL). We used 1 mg/mL HA-FL as it corresponds to 0.1%, which is used in commercial eye drops. The mice were then sacrificed at the 3 different time points: mice 1 and 2 were sacrificed at 0 min right after eye drop application, and mice 3-6 were allowed to regain consciousness as isoflurane gas was removed, and then they were sacrificed at either 15 min or 30 min after eye drop application. Whole mice were imaged immediately after sacrificing using a fluorescent dissecting microscope (Nikon, Melville, NY), allowing us to take images of the eye without harvesting the tissues.

3. RESULTS

3.1 NMR Results

After a multi-step synthesis of SABpep-PEG/Succ-CD-HABpep, NMR was used to characterize it to confirm that the actual product synthesized is SABpep-PEG/Succ-CD-HABpep. **Figure 5** depicts the NMR results for PEG/Succ-CD in DMSO (**Figure 5A**), PEG/Succ-CD/Acrylate in D_2O (**Figure 5B**), SABpep in D_2O (**Figure 5C**), HABpep in D_2O (**Figure 5D**), PEG/Succ-CD-SABpep in D_2O (**Figure 5E**), and SABpep-PEG/Succ-CD-HABpep in D_2O (**Figure 5F**). ^{1}H NMR (400 MHz, D_2O): δ 3.5-3.7 (4H, PEG), 4.9-5.1 (6H, succ-CD), 6.7-6.9 (3H, SABpep), and 8.4-8.6 (16H, HABpep).

In order to identify the ratio of PEG to Succ-CD to SABpep to HABpep to determine the valency of the product, the NMR peaks corresponding to the number of hydrogens of each were integrated. The 4H in PEG (from -CH_2-CH_2) at ~3.6 ppm corresponds to a signal of 5.1269 units. The 6H in Succ-CD (from –OH) at 5.0 ppm corresponds to a signal of 1.7078 units. The 3H in SABpep at 6.8 ppm corresponds to a signal of 0.2300. And the 16H in HABpep at 8.5 ppm corresponds to a signal of 0.6784. From these integrations, the ratio of each can then be calculated as follows:

PEG (M_w 20,000 g/mol with repeat unit, n = 44 g/mol; 4H present in signal of 5.1269; x = relative number of PEG chains;):

$$44 * n = 20{,}000$$

$$\therefore\ n = 454\ repeat\ units$$

$$x * (454 * 4) = 5.1269$$

$$\therefore\ x = 0.0028$$

Succ-CD (6H present in signal of 1.7078; y = relative number of Succ-CD):

$$Maximum\ number\ of\ Succ-CD\ molecules\ on\ each\ PEG\ chain = \frac{n}{2} = \frac{454}{2} = 227$$

$$y * 6 = 1.7078$$

$$\therefore\ y = 0.2846$$

SABpep (3H present in signal of 0.2300; z_1 = relative number of SABpep):

$$z_1 * 3 = 0.2300$$

$$\therefore\ z_1 = 0.07667$$

HABpep (16H present in signal of 0.6784; z_2 = relative number of HABpep):

$$z_2 * 16 = 0.6784$$

$$\therefore\ z_2 = 0.0424$$

Ratio of PEG to Succ-CD to SABpep to HABpep:

$$PEG = 0.00282318 = 1$$

$$Succ-CD = 0.2846 \cong 100$$

$$SABpep = 0.07667 \cong 27$$

$$HABpep = 0.0424 \cong 15$$

$$Total\ valency = SABpep + HABpep = 42$$

Thus, from the NMR results, for every PEG chain, there are approximately 100 succ-CD molecules threaded on it, with approximately 27 SABpep and 15 HABpep attached. This demonstrates that the multivalent SABpep-PEG/Succ-CD-HABpep system has approximately 42 arms.

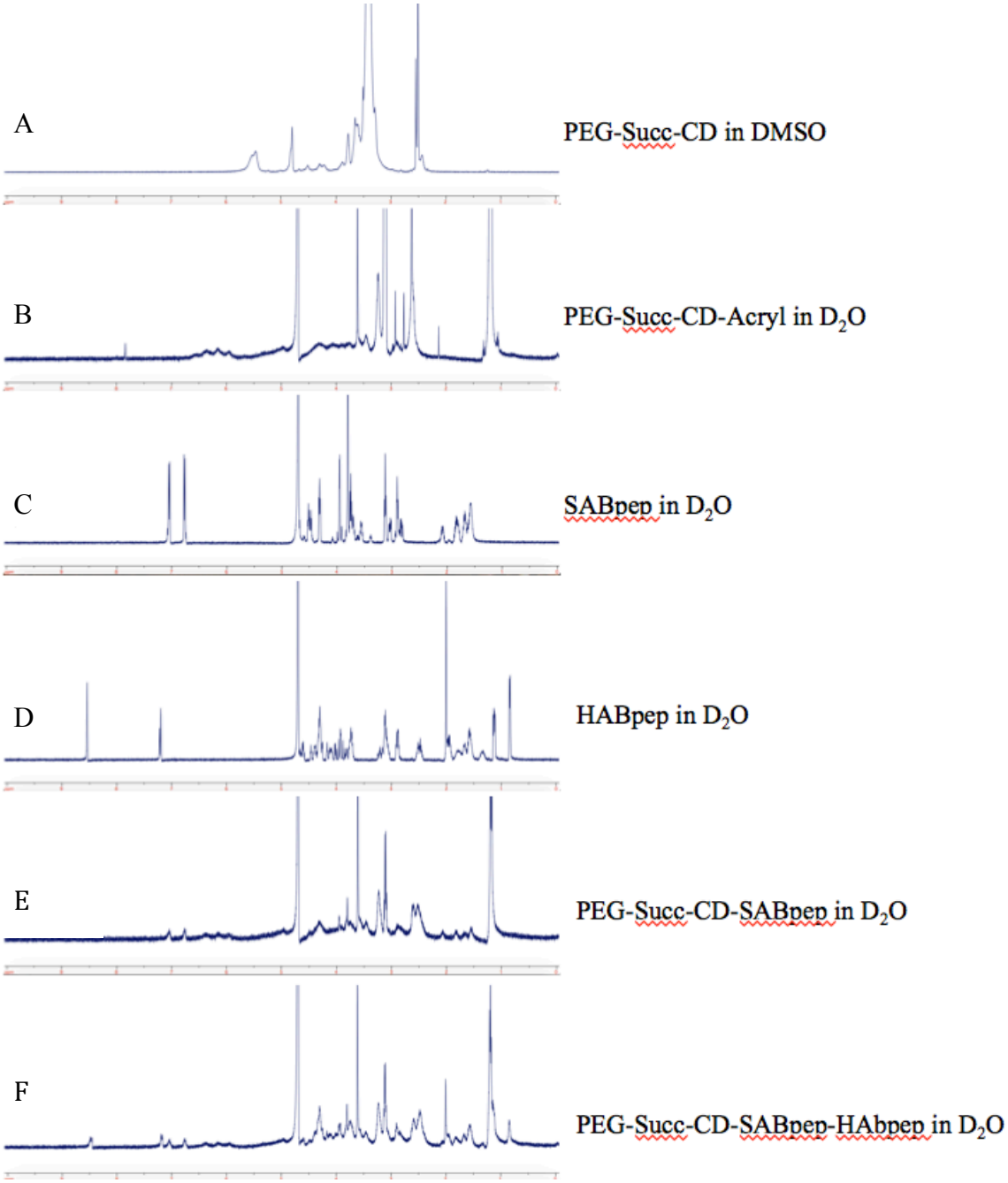


FIGURE 5. ^{1}H-NMR of alpha-CD threaded on PEG chains with SABpep and HABepep.

3.2 *Ex vivo* binding of PEG/Succ-CD/SABpep and PEG/Succ-CD/SABpep/HABpep on conjunctival tissue

The *ex vivo* rabbit eye study was done to examine the retention of HA of different eye drop solutions on *ex vivo* rabbit eyes over time, with each wash representing blinking of the eyes. The fluorescent value of each condition was measured after each wash with a total of 15 washes, and the eye drop was re-applied 2 more times, each with 15 washes. Each wash corresponds to 5 min, so these fluorescent values were then converted to concentration of HA-FL over time using a standard HA-FL curve, and then the percent of HA retained after each wash (or after every 5 min) was plotted against time. **Figure 6** shows the percent of HA retained over time comparing the control (which is the baseline with PBS + HA-FL), the SABpep-PEG/CD + HA-FL, the SABpep-PEG/CD-HABpep + HA-FL, and the 8-arm SABpep-PEG-HABpep + HA-FL.

After the first wash (5 min), the SABpep-PEG/CD-HABpep retained 40% HA, the 8-arm SABpep-PEG-HABpep retained 33%, the SABpep-PEG/CD retained 29%, and the control (HA-FL only) retained 28%, with respect to the initial signal at time = 0 min, where the SABpep-PEG/CD-HABpep retained 51% HA, the 8-arm SABpep-PEG-HABpep retained 42%, the SABpep-PEG/CD retained 40%, and the control (PBS + HA-FL only) retained 43%. This demonstrates that after 5 min of eye drop application, SABpep-PEG/CD-HABpep retained ~1.2 times more HA than 8-arm SABpep-PEG-HABpep, ~1.4 times more HA than SABpep-PEG/CD, and ~1.4 times more HA than the control.

After 1 period of eye drop treatment (75 min or 1.25 h), the SABpep-PEG/CD-HABpep retained 22% HA, the 8-arm SABpep-PEG-HABpep retained 18%, the

SABpep-PEG/CD retained 17%, and the control (PBS + HA-FL only) retained 15%. After the second period of eye drop treatment (195 min or 3.25 h), the SABpep-PEG/CD-HABpep retained 20% HA, the 8-arm SABpep-PEG-HABpep retained 16%, the SABpep-PEG/CD retained 15%, and the control (PBS + HA-FL only) retained 13%. This second period followed the same trend as the first period where after each period, SABpep-PEG/CD-HABpep retained ~1.2 times more HA than 8-arm SABpep-PEG-HABpep, ~1.3 times more HA than SABpep-PEG/CD, and ~1.5 times more HA than the control.

After both the first and second periods of eye drop treatment (or 195 min) with about 75 min each period with 40 min in between for eye drop application, the SABpep-PEG/CD-HABpep + HA-FL demonstrated the greatest retention of HA on the eye. This emphasizes that the HA binding eye drop technology with the PEG/Succinyl-CD polymer, SABpep, and HABpep has the greatest potential in increasing the retention time of HA on the ocular surface. This is due to the multivalency of the PEG/CD complex, which allows it to bind more HA, as compared to the control and other polymer-peptide systems.

However, the third period of eye treatment (after 315 min or 5.25 h) shows a different trend where the SABpep-PEG/CD-HABpep retained 20% (which is the same as after the second period), but the 8-arm SABpep-PEG-HABpep retained 21%, the SABpep-PEG/CD retained 17%, and the control retained 20%. This could be due to the fact that the eye drops only have significant or accurate residence time before 200 min. After 200 min, there is no significant difference among groups and controls.

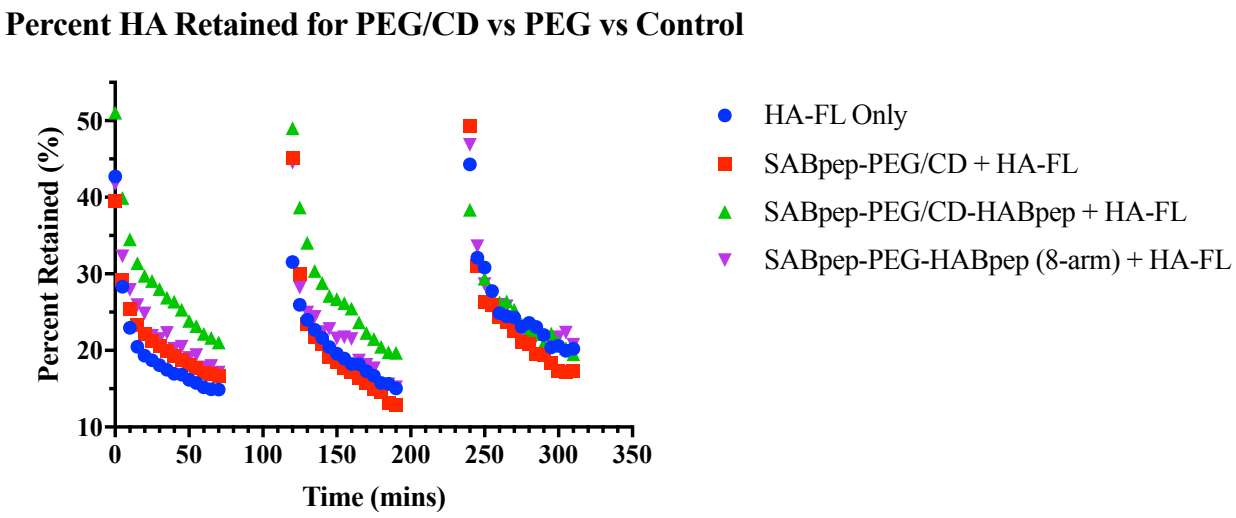


FIGURE 6. *Ex vivo* rabbit model study: Percent HA retained over time for the control (HA-FL only), PEG/CD bound only to SABpep with HA-FL, PEG/CD bound to both SABpep and HABpep with HA-FL, and 8-arm PEG bound to both SABpep and HABpep with HA-FL. 1st Graph-1st batch of application, 2nd graph-2nd batch of application, 3rd graph-3rd batch of application.

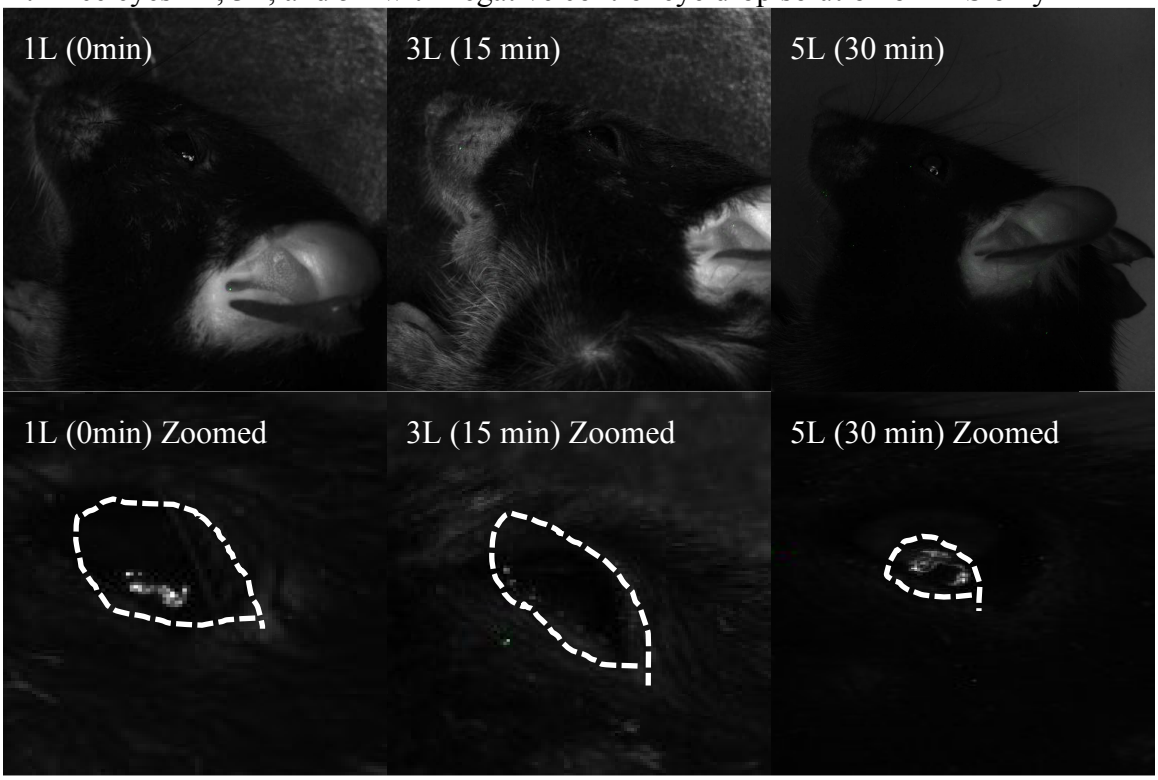
3.3 *In vivo* binding of PEG/Succ-CD/SABpep/HABpep on mice

The *in vivo* HA retention study had 4 different eye drop solutions that were compared at 3 different time points. The first eye drop solution was a negative control of PBS only (without HA-FL) (**Figure 7A**). The second eye drop solution was a negative control of SABpep-PEG/CD-HABpep only (**Figure 7B**). The third eye drop solution contained an equal volume mixture of PBS and 0.1% HA (as in commercial HA eye drops) (**Figure 7C**). The fourth eye drop solution contained an equal volume mixture of SABpep-PEG/CD-HABpep and 0.1% HA (**Figure 7D**). Each mouse eye that was imaged has a zoomed-in image below to show the details of the fluorescent intensity of the eye drop solution. **Figures 7A and 7B** show that the negative control eye drop solutions (without HA-FL) show no fluorescence in the mice eyes at 0 min, 15 min, or 30 min.

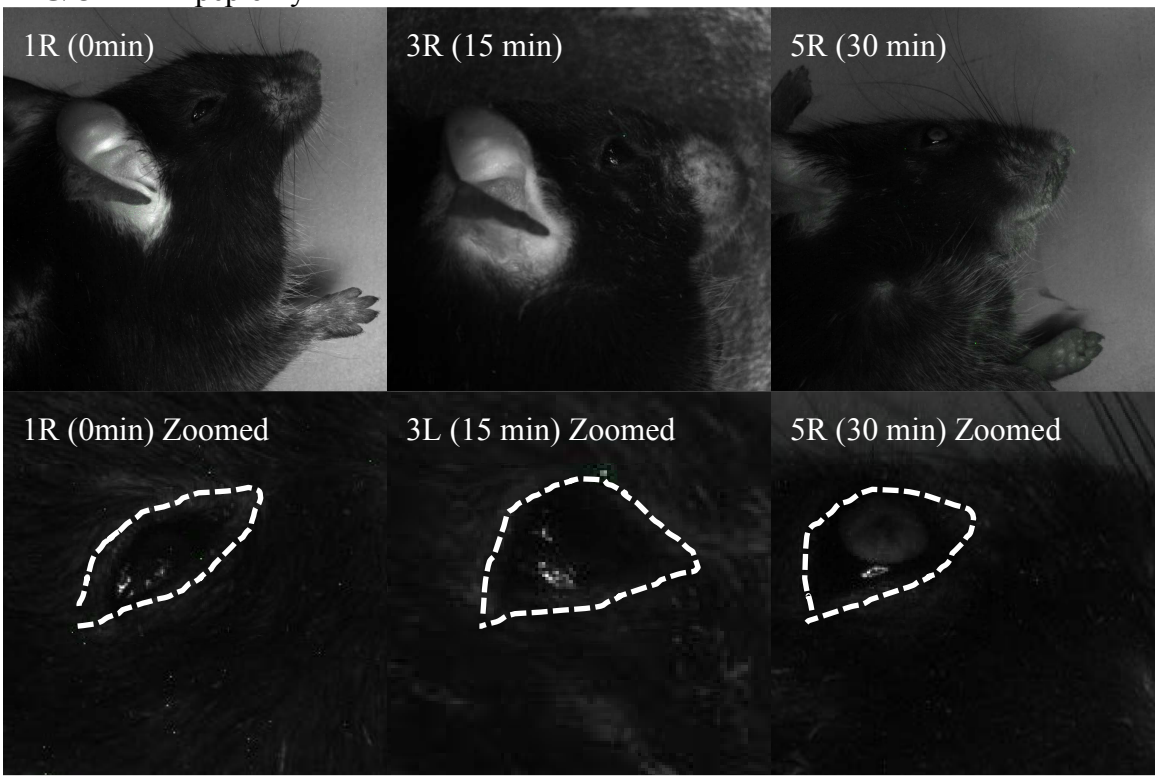
This confirms our hypothesis since no HA-FL eye drop solution was added, so there should be no fluorescence in the mice eyes. The visible white spots that appear in these two figures are due to reflection of light from the eyes. **Figures 7C and 7D** depict that both the positive control and the sample eye drop solutions showed high, uniform fluorescence at 0 min. However, over time, the positive control eye drop solution was quickly vanishing and showed much less fluorescent intensity at 15 min, compared to the sample eye drop solution, and it showed no fluorescent intensity at 30 min, while the fluorescence of the sample eye drop solution was still present. To clarify it further, the 30 min positive control eye (**Figure 7C**) shows no fluorescence; however, a fluorescent spot appears to be on the eyelid and not on the eye, and could be due to blinking of the eyes; thus, the positive control eye shows no fluorescence at 30 min. Also, at 15 min for **Figures 7C and 7D** for the positive control and sample eye drop solutions, respectively, they both exhibit fluorescence; however, the sample eye drop solution in **Figure 7D** shows a uniform layer of the solution on the eye, whereas the positive control solution in **Figure 7C** shows a thin fluorescent band of the solution at the bottom of the eye, possibly at the edge of the eye lid. Both eyes experienced blinking, but multivalent polymer-peptide, SABpep-PEG/CD-HABpep + HA-FL (sample) was able to retain the HA uniformly on the eye, whereas the positive control of PBS + HA-FL was not. This shows that the SABpep-PEG/CD-HABpep + HA-FL lasted well on the ocular surface at 15 min and was still present at 30 min, whereas the control only lasted until 15 min but was not uniform and did not cover the surface of the eye. Initial and over time fluorescence of the positive control and sample eye drop solutions remained

approximately the same, so this rules out the difference in fluorescence values due to pipetting errors.

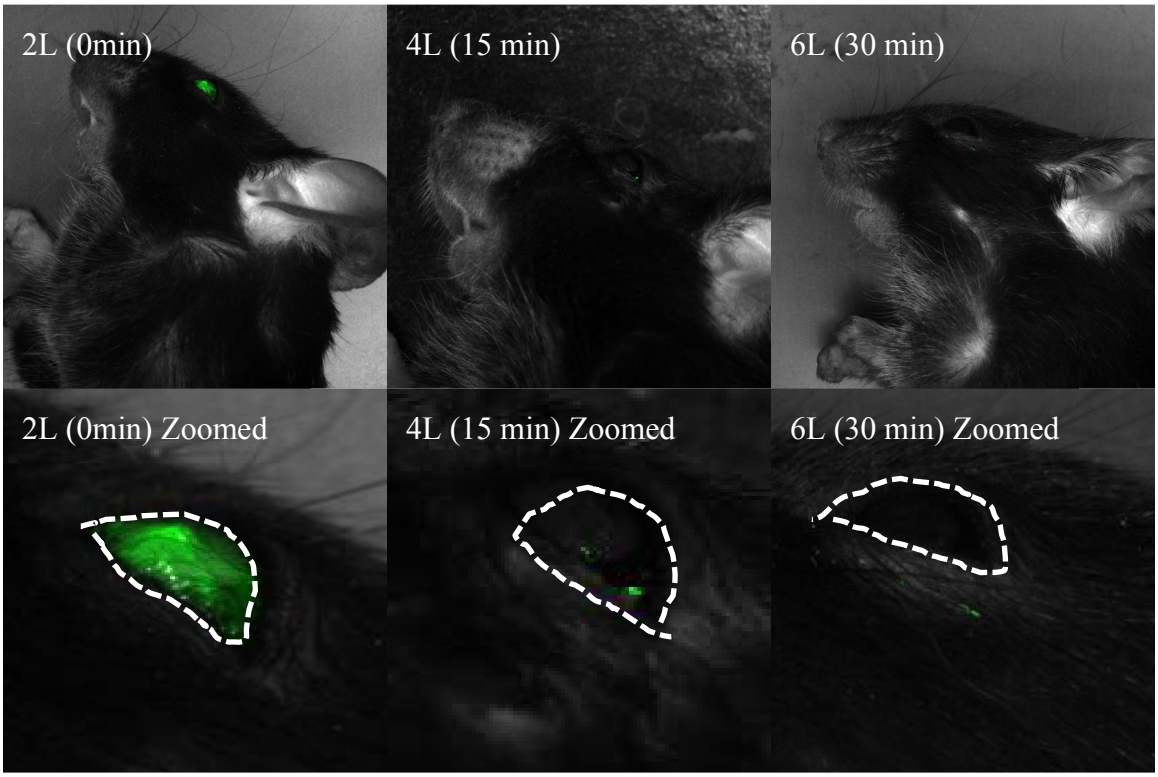
A. Mice eyes 1L, 3L, and 5L with negative control eye drop solution of PBS only



B. Mice eyes 1R, 3R, and 5R with negative control eye drop solution of SABpep-PEG/CD-HABpep only



C. Mice eyes 2L, 4L, and 6L with positive control eye drop solution of PBS + 0.1% HA-FL



D. Mice eyes 2L, 4L, and 6L with sample eye drop solution of SABpep-PEG/CD-HABpep + 0.1% HA-FL

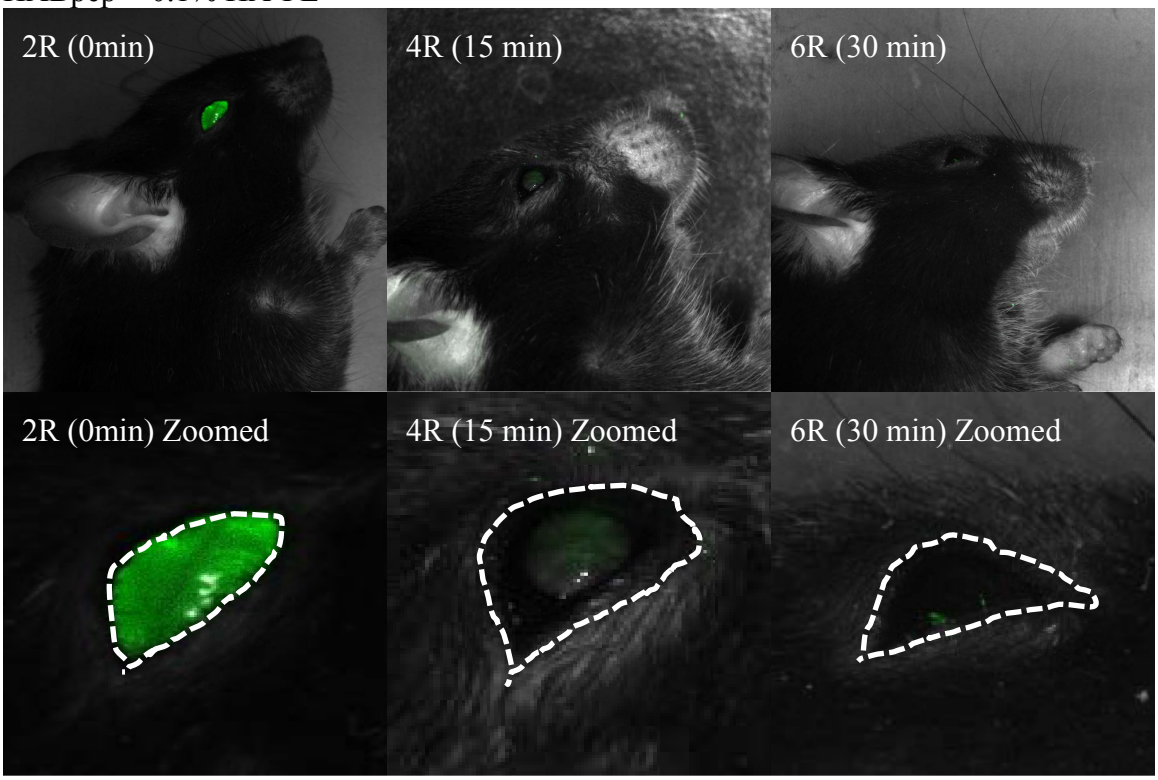


FIGURE 7. Fluorescent images of *in vivo* eye drop treatment in mice eyes. The figure is divided up into 4 sets, where each set shows zoomed-in images of each eye. **A** compares the negative control mice eyes 1L (mouse 1, left eye), 3L, and 5L where only PBS was applied at different time points t = 0 min, 15 min, and 30 min, respectively. **B** compares the negative control mice eyes 1R, 3R, and 5R where only the multivalent polymer-peptide was applied (SABpep-PEG/CD-HABpep) at different time points t = 0 min, 15 min, and 30 min, respectively. **C** compares the positive control mice eyes 2L, 4L, and 6L where PBS and HA-FL were applied at different time points t = 0 min, 15 min, and 30 min, respectively. **D** compares the sample mice eyes 2R, 4R, and 6R where SABpep-PEG/CD-HABpep and HA-FL were applied at different time points t = 0 min, 15 min, and 30 min, respectively.

4. DISCUSSION

Eye drops have become a conventional form for ocular therapy, and they now represents 90% of the market for ocular conditions due to their simplicity of development and production.[23] It has been shown through studies mentioned previously that incorporating hyaluronic acid in these eye drops helps improve ocular residence time of these eye drops. Thus, there are several eye drop products in the market[7], shown in **Figure 8**. The commercially available eye drops containing 0.1-0.2% hyaluronic acid (HA) solutions, such as Opticalm™, Scope™, and Oxyal™ are commonly used as an active ingredient in Europe to relieve dry eye symptoms, while in USA as an inactive ingredient of the eye drop formulation (e.g. in Blink™). **Figure 8** shows 10 different types of eye drops and their status of FDA approval. The only eye drop product that is FDA approved and sold in the US market is Blink™, where its main component is 0.25% PEG and HA is listed as an inactive ingredient. One of the disadvantages of these products is that they show low ocular residence time since these current eye drops reside in the aqueous layer of the tear film on the ocular surface, so after some blinking, the eye drop is easily washed away.

Product (Company)	Active Ingredient	Other Information	FDA Approval Status
Vismed (TRB Cemedica)	0.18% Sodium Hyaluronate	Preservative free	Phase III completed, marketed in UK, Europe and Asia
Vislube (TRB Cemedica)	0.18% SH solution	Preservative free	Marketed in Europe, Australia and Asia
Hylabak or Hyabak (Spectrum Thea Pharmaceuticals)	0.15% Hyaluronic Acid or SH	Preservative free Phosphate free	Marketed in Russia, Lebanon, Argentina, and Germany
Hyalein	0.1% SH	Active control in Phase III study	Phase III completed, marketed in Japan
Opticalm (Omega Pharma)	0.2% SH	Also contains 0.2% Hypromellose	Marketed in Europe
Hylovis (TRB Cemedica)	0.18% SH	Preservative free	Marketed in Europe, Australia, and Asia
Blink Tears (Abbott)	0.25% Polyethylene Glycol	HA listed as inactive ingredient	Approved: Phase IV completed
Rejena (sponsor)	0.18% HA	Data considered weak	Not approved by FDA
JDE-001 (Jade Therapeutics)	Thiolated carboxy-methyl HA	Designed to improve tear-film stability	Pre-IND Stage
SI-614 Ophthalmic-Solution (Seikagaku)	Modified HA	Possibly improves residence time and tear-film stability	Phase III ongoing

FIGURE 8. HA and modified-HA artificial tear products.

The novel idea of the polymer-peptide system is that it immobilizes the HA onto the mucin layer of the ocular surface, allowing for enhanced binding as opposed to having HA in the aqueous layer of the tear film. Theoretically, since HA is immobilized onto the ocular surface with this system, it should be able to reside on the ocular surface

for a longer amount of time since the bond is supposedly stronger so the blinking should not affect the eye drop as much, especially in the earlier time points.

In previous studies done by Lee el al.[25], they found that immobilizing HA using an HABpep by developing a heterobifunctional polymer-peptide system with SABpep at the other end of the spacer PEG molecule showed that HA was bound 1.8 times more in the beginning and ~1.2 times more at 24 h through 2-arm SABpep-PEG-HABpep compared to control (HA only), through their *ex vivo* rabbit experiment. They also found that through their *in vivo* mouse model, the heterobifunctional polymer-peptide system (2-arm PEG) was observed even at 15 min compared to 5 min for the control HA that was not bound with the peptide.

In comparison, this novel PEG/Succ-CD complex with multivalent binding of SABpep and HABpep to SA and HA, respectively, in the *ex vivo* rabbit model demonstrated that it initially retained ~1.2 times more HA than 8-arm SABpep-PEG-HABpep, ~1.4 times more HA than SABpep-PEG/CD, and ~1.4 times more HA than the control. And after period 1 and 2 of eye drop application, or 75 min or 195 min, the novel SABpep-PEG/CD-HABpep retained ~1.2 times more HA than 8-arm SABpep-PEG-HABpep, ~1.3 times more HA than SABpep-PEG/CD, and ~1.5 times more HA than the control. This shows that this multivalent polymer-peptide system has higher binding of HA to the ocular surface than the 8-arm polymer-peptide system, as well as the control (without the peptide). These results, however, cannot show higher binding of HA to the ocular surface than the heterobifunctional polymer-peptide system (2-arm) that was produced in the study done by Lee et al[25], since the experimental setup and methods were different. But theoretically, this multivalent polymer-peptide system should show higher

retention of HA to the ocular surface than the 2-arm polymer-peptide system, since the number of arms/valency is higher.

In the *in vivo* mouse model, the novel eye drop technology of a multivalent polymer-peptide system (SABpep-PEG/CD-HABpep + HA-FL) showed that this eye drop solution lasted up to 30 min, while the positive control containing PBS + HA-FL only lasted up to 15 min. Both these eye drop solutions showed high initial fluorescence at time t = 0 min; however, the control was quickly vanishing and even after 15 min, it was almost depleted, but after 30 min, only the SABpep-PEG/CD-HABpep + HA-FL eye drop was retained on the mouse eye. Thus, the preliminary results from this model emphasized that the multivalent polymer-peptide eye drop had a residence time of ≤ 30 minutes, as compared to ≤ 15 minutes for the heterobifunctional polymer-peptide eye drops (2-arm PEG) and for the commercially-available HA-based eye drops. This shows that the novel eye drop technology does have great potential to be an effective treatment method for dry eye disease.

5. CONCLUSION

In summary, we developed a new eye drop technology using a multivalent polymer-peptide system that immobilizes HA onto the ocular surface using sialic acid as an anchor. The retention time of this eye drop technology was significantly improved compared to previous technologies, as it retained ~1.5 times more HA than commercially-available eye drops through *ex vivo* rabbit eye studies and had a residence time of ≤ 30 minutes through *in vivo* mice eye studies, as compared to ≤ 15 minutes for previous eye drop technologies. Even though other pre-clinical tests are needed, we anticipate that this HA eye drop would reduce the frequency of eye-drop instillation and also more effectively alleviate dry eye symptoms by improving water retention.

6. FUTURE DIRECTIONS

The *in vivo* mouse model done in this study showed preliminary results that imply the great potential of this multivalent polymer-peptide system to increase HA retention time on the ocular surface. However, this *in vivo* test should be repeated to confirm these results and to compare this eye drop technology to the 8-arm SABpep-PEG-HABpep and the 2-arm SABpep-PEG-HABpep technologies, to investigate if this eye drop can retain HA for 30 minutes, which should be longer than the other eye drops.

We are currently performing isothermal titration calorimetry (ITC) protocol to explore the binding affinity of HA to 2-arm x-PEG-HABpep, 8-arm PEG-HABpep, and multivalent PEG/Succ-CD/HABpep, as well as the binding affinity of mucin (or sialic acid) to 2-arm PEG-SABpep, 8-arm PEG-SABpep, and multivalent PEG/Succ-CD/SABpep. We hypothesize that the binding affinity of the multivalent PEG/CD complex would have a higher binding affinity to both HA and mucin than the 8-arm PEG, which would have a higher binding affinity than the 2-arm PEG. This is because more arms/branching/valency should indicate a higher binding affinity. Once this hypothesis is confirmed, then we can run ITC to explore the binding affinity of HA and mucin to the full polymer-peptides systems: 2-arm SABpep-PEG-HABpep, 8-arm SABpep-PEG-HABpep, and SABpep-PEG/Succ-CD-HABpep. As mentioned previously, we hypothesize that SABpep-PEG/Succ-CD-HABpep would have a higher binding affinity to both HA and mucin than the 8-arm SABpep-PEG-HABpep, which would have a higher binding affinity than the 2-arm SABpep-PEG-HABpep, due to increasing number of arms/valency.

In a future test, we would like to run ocular biomechanical friction test with our collaborator (Prof. Tannin A. Schmidt's laboratory) at the University of Connecticut. We hypothesize that rabbit ocular tissues treated with our multivalent polymer-peptide system (SABpep-PEG/CD-HABpep) and HA solution would have reduced kinetic coefficient of friction values, and therefore improved tissue lubrication, compared to HA-only treated tissues as control and the 2 other heterofunctional eye drops (2-arm SABpep-PEG-HABpep and 8-arm SABpep-PEG-HABpep).

A future direction for this project could be implementing dendrimers to allow even more HABpep and SABpep to bind, potentially increasing HA retention time even more, or, enhance the multi-valency in the present system. Dendrimers are star-shaped macromolecules that are highly branched, where they also display multivalency. This could be another method to improve HA retention on the ocular surface as an effective treatment method for dry eye disease.

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CURRICULUM VITAE

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Education

Johns Hopkins University, Baltimore, MD August 2016 – May 2018
Master of Science in Biomedical Engineering

- Major: Biomedical Engineering with a focus in tissue engineering & biomaterials/polymers
- Relevant coursework: Polymer Design & Bioconjugation, Intro to Polymeric Materials, Biomolecular Materials, Chemistry of Materials Synthesis, Polymer Physics, Physical Epigenetics, Design of Biomolecular Systems
- Activities: Public Relations Chair of Women of Whiting, Graduate Advisor of Musicare

Johns Hopkins University, Baltimore, MD August 2012 – May 2016
Bachelor of Science in Biomedical Engineering

- Major: Biomedical Engineering with a focus in cell & tissue engineering
- Relevant coursework: BME Modeling & Design, Systems Bioengineering I-III & Labs, Models & Simulations, Signals, Systems, & Controls, Statistical Mechanics, & Thermodynamics, Cell Engineering, Tissue Engineering, Materials Characterization, Intro to Business, Intro to Programming: Java, MATLAB
- Activities: President of Musicare, Student Advocacy Board member for Center for Social Concern, Member of ABC Health, Member of JHU Golf, Volunteer at Shepherd's Clinic
- Extracurricular interests: Violin, golf, tennis

Relevant Experience

Johns Hopkins University, Baltimore, MD August 2016 – Present
Master's Student Researcher, Department of Urology, Tissue & Chemical Engineering Research Lab

- Develop a hyaluronic acid (HA) binding eye drop technology that prolongs HA retention on the ocular surface as a treatment method for dry eye disease
    - Synthesize a polymer-peptide system for the eye drop and test its retention on *ex vivo* rabbit eyes
- Create a biomaterial using modified collagen for an artificial bladder for bladder cancer patients
    - Modify collagen through chemical reactions
    - Characterize the material made using NMR, FTIR, DSC, SEM, TEM, XRD, and Circular Dichroism
    - Test the mechanical properties of the material using suture, tensile testing, & rheology/viscosity testing
- Submitting a manuscript to the Journal of Tissue Engineering

National Taiwan University, Taipei, Taiwan July – August 2016
Summer Student Researcher, Department of Biomedical Engineering, Biomechanics Research Lab

- Studied the effect of ultrasound stimulation on the convection of molecular particles within porous media to see if ultrasound stimulation can help induce disc regeneration for intervertebral disc degeneration (IVD)
- Used gelatin and Fluorescein Sodium (FS) particles to simulate the diffusion of molecular particles within porous media and seeing the effect of ultrasound stimulation on this process using fluorescent imaging
- Submitted abstract to the Orthopaedic Research Society (ORS) in August 2016

Johns Hopkins University, Baltimore, MD August 2015 – May 2016
Design Team Member, Non-Invasive Device for Parkinson's Patients

- Developed a device that used transcranial Direct Current Stimulation (tDCS) to help alleviate symptoms, such as hand tremors, for Parkinson's patients in a home setting
- Gained skills including ideating, prototyping, collaborating, clinical testing, and presenting
- Placed 3rd at the Johns Hopkins University Business Plan Competition in April 2016

Johns Hopkins Medical Institute, Baltimore, MD February 2014 – May 2015
Design Team Member/Researcher, Neonatal Infant Resuscitation Head-tilt Angle Study

- Determined the head-tilt/sniffing angle for open airway in neonatal infants for maximizing neonatal resuscitation in developing countries
- Processed MRI images and analyzed patient data records
- Published a paper in the peer-reviewed journal, PLOS One

University of Colorado Anschutz Campus, Aurora, CO Summers of 2014 – 2015
Volunteer Research Assistant, Center of Hypoxia and Inflammation Research

- Assisted a postdoc with research on acute lung injury and inflammation
- Gained skills in cell culture, RNA isolation from tissues, RT-PCR, and Western blots to look at protein expression

Publications/Manuscripts

Anirudha Singh, David Lee, **Christine Yu**, Harrison Jeong, Praveena Sabnekar, Xiaopu Liu, Takahiro Yoshida, Nikolai Sopko, Trinity J. Bivalacqua. (2017). Tissue-engineered Neo-Urinary Conduit from Decellularized Trachea. *Tissue Engineering*. *Manuscript submitted.*

Utpal S. Bhalala, Malvi Hemani, Meehir Shah, Barbara Kim, Brian Gu, Angelo Cruz, Priya Arunachalam, Elli Tian, **Christine Yu**, et al. (2016). Defining optimal head-tilt position of resuscitation in neonates and young infants using magnetic resonance imaging data. *PloS one*, *11*(3), e0151789.